Effects of High-Heat Treatment on Stability of Calcium Caseinate Aggregates in Milk

2599

K. K. FOX, M. K. HARPER, V. H. HOLSINGER, and M. J. PALLANSCH

Dairy Products Laboratory
Eastern Utilization Research and Development Division, USDA, Washington, D. C.

Abstract

Under standard conditions of centrifugation, the concentration and composition of the nonsedimenting nitrogen content of heated milks are found to vary. It decreases in concentration with increasing temperature to between 103 and 110 C, where its concentration increased again with temperature. Below 103 C the nonsedimenting nitrogen fraction was found to consist of whey proteins and the proteose-peptone fraction; at 110 C and above the nonsedimenting nitrogen fraction consisted predominantly of caseinate. The amounts of nonsedimenting nitrogen produced above 110 C was dependent on the temperature of heating, the concentration of milk solids, and on the phosphate and citrate concentrations. It has been reported by others, and also found in this investigation, that the soluble calcium and phosphate concentrations of milk decreased during heating, whereas those of citrate remained constant. Soon after heating, the soluble calcium concentration of milk returned to its former value. Formation of dissociated caseinate at temperatures of 110 C and above may be attributed to removal of calcium from caseinate aggregates by the free citrate ion. Since the citrate concentration was not changed by heating, its formation of a soluble calcium citrate complex also returns the soluble calcium concentration to its value before heating.

Experiments to determine the effect of different heat treatments on the amount and kind of nitrogen remaining in the supernatant liquids of centrifuged milk have been conducted by a number of investigators. The principal concern of these investigators appears to have been the fate of the whey proteins in heated milk. Ramsdell and Whittier (10) analyzed the centrifuge supernatant of heated milk for acid-coagulable nitrogen. They concluded that heating skimmilk for 10 min at 212 F produced no measurable aggregation of the whey proteins. Edmondson and Tarassuk (3), on the basis of somewhat similar experiments, pointed out that denatured whey proteins might sediment in the same manner as casein. Sullivan et al. (14) noted that any increase in centrifugable nitrogen beyond 78% of the total nitrogen would indicate that denatured whey proteins were being sedimented. These experiments all showed a decrease in the supernatant nitrogen of heated milks relative to that in raw milk. Only Ramsdell and Hufnagel (9) appear to have noted a decrease in particle size of the caseinate system when it was heated. In this laboratory it was observed that the amount of nitrogen sedimented from conventional evaporated milks by centrifuging was always less than 60% of the total nitrogen, which is considerably less than the approximately 75% sedimentable nitrogen found in unheated milk.

Objectives of the research reported in this paper were to ascertain the factors responsible for the occurrence of the nonsedimentable nitrogen in sterile milk concentrates, and to account for the difference in amount of nonsedimentable nitrogen found in evaporated milks and the experimental results reported in the literature as noted above.

Experimental Procedures[1]

Centrifuging was done in a Spinco Model L centrifuge, using a no. 40 rotor. All milks, including concentrated milks, were centrifuged at room temperature (20-22 C) at 105,000 × g (avg) for 30 min. After such centrifugation, there exists a turbid volume of about 1 ml lying next to the caseinate pellet, which can be readily discerned by the sharp concentration gradient separating it from the solution above. Analysis of the overlying solution with increasing depth in the centrifuge tube, but not including the described turbid volume, showed it to be quite homogeneous with respect to nitrogen distribution. The nitrogen in the overlying solution is termed nonsedimenting nitrogen (NSN).

---

[1] Reference to certain products or companies does not imply an endorsement by the Department over others not mentioned.

Nitrogen analyses were made by a standard micro-Kjeldahl procedure (1). The calcium concentration of solutions containing protein was determined from the ash of an aliquot of the solution by a back titration method using calcein as the indicator (15). Phosphorus was determined on the same ashed sample by the method of Fiske and Subbarow (5), using a Bausch and Lomb Spectronic 20 Colorimeter. Citrate was determined by a colorimetric procedure, based on the interaction of citrate with pyridine and acetic anhydride (8).

Heating of milk samples and of milk dialysate to temperatures up to 100 C was done in a thermostated water bath, which maintained the temperature to ± 0.5 C. The samples were rapidly brought to the desired temperature by immersion in a boiling-water bath until within 2-3 C of the desired temperature, then transferred to the thermostated water bath. After heating, the samples were immediately cooled to room temperature in an ice-water mixture.

Dialysis of completely decalcified milk against normal milk was done as follows: Calcium was removed from a sample of milk by precipitation with a slight excess of neutral oxalate. The clarified solution obtained by removing the precipitated CaC_2O_4 by centrifuging 10 min at 26,000 $\times g$ (avg) was placed in cellophane dialysis tubing and dialyzed vs. milk at 4 C. After 4, 18, and 24 hr of dialysis, the solution was again centrifuged and the dialysis tubing replaced. This was done to prevent interference with dialysis by precipitation of calcium oxalate in or on the walls of the dialysis tubing.

The concentration of calcium, phosphorus, and citrate ion in milk dialysate and in a synthetic sera after heating was determined as follows: After heating for 10 min at the different temperatures, the hot solutions were filtered through Whatman no. 42 filter paper. The hot filtrate was cooled in an ice bath while being collected. Cans of dialysate and synthetic sera heated to 118 C in a pilot sterilizer were opened and their contents filtered immediately after the cans were removed from the sterilizer. Analyses for the separate ions were made by the procedures already described.

To determine the effect of concentration of milk solids on the production of nonsedimenting nitrogen, a solids-not-fat (SNF) concentrate of 44.3% total solids was obtained from the laboratory pilot plant. The sample was an aliquot of a large batch of such concentrate which had been prepared from pasteurized skimmilk (74 C, 16 sec) by two passes through a falling-film evaporator. The concentrate was diluted to different concentrations and these

left to equilibrate for 20 hr at 20-22 C before centrifuging or sterilizing.

Milk dialysate was obtained by dialyzing 500 ml of water to equilibrium against 40 liters raw milk at 4 C.

In experiments involving addition of phosphate and citrate, the phosphate was an equimolar mixture of K_2HPO_4 and KH_2PO_4, and the citrate was the hydrated trisodium salt. Twenty-hour equilibration of the samples containing added salts at 20-22 C was allowed before sterilization or centrifugation.

Disc electrophoresis was carried out on poly-acrilamide gel, using a Canalco apparatus as recommended by the manufacturer.

With the exception of the evaporated milks examined originally, the experiments reported have been conducted with concentrates of solids-not-fat. This was done because the fat-protein complexes in the homogenized products complicate interpretation of the results. Depending on the fat : protein ratio and density of the solution, the complexes either sediment, remain in the supernatant, or float when centrifuged.

Results

Effect of temperature on the concentration of NSN in milk samples heated for 30 min is shown in Table 1. It is seen that the concentration of NSN falls with increasing temperature up to about 90 C. Above this temperature the concentration of NSN increases with increasing temperature within the temperature range covered.

In Figure 1 are shown the disc electrophoretic patterns of milks heated to different temperatures for 30 min. Temperatures range from 70 to 117 C. Identification of the different components in the NSN, present at different

TABLE 1

Concentration of nonsedimenting nitrogen in milk samples heated to different temperatures for 30 min

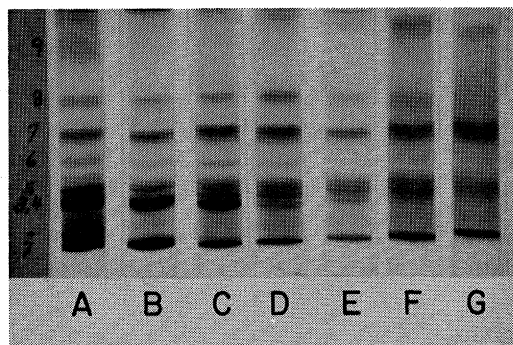| Temperature	Total nitrogen as nonsedimenting nitrogen
(C)	(%)
Control (not heated)	27.3
70	25.7
75	21.0
80	18.8
85	18.8
90	17.5
94	18.1
103	18.2
110	22.5
117	28.9

Fig. 1. Disc electrophoretic patterns of the non-sedimenting nitrogen fractions of milk samples heated for 30 min at:

    A. Not heated
    B. 75 C
    C. 80 C
    D. 85 C
    E. 94 C
    F. 110 C
    G. 117 C

temperatures, was attempted by means of the electrophoretic patterns of pure components shown in Figure 2. Numbers on the left side of the figures are for the purpose of distinguishing different components and not for indicating their total number. Protein components of whey and casein, identified by reference to relatively pure preparations of these components, are listed in Table 2.

By reference to Figure 1 it may be seen that the immune globulins are the most heat-labile components of the NSN, as shown by their disappearing first from the electrophoretic pattern of NSN. Components 6, 7, and 8 are
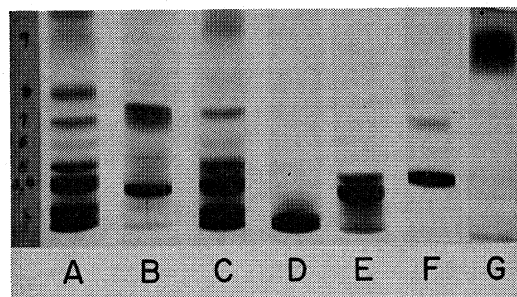

Fig. 2. Disc electrophoretic patterns of fractions and relatively pure preparations of individual proteins found in the nonsedimenting nitrogen fraction of milk samples heated to different temperatures:

    A. NSN fraction from unheated milk
    B. Casein fraction
    C. Whey protein fraction
    D. β-Lactoglobulin
    E. α-Lactalbumin
    F. Serum albumin
    G. Immune globulins

TABLE 2

Identity of components in the nonsedimenting nitrogen fraction of milk samples heated to different temperatures. Numbers are those used in Figures 1 and 2

Component no.	Protein component
1	Tracking boundary
2	β-Lactoglobulin
3	α-Lactalbumin
4	β-Casein
5	Bovine serum albumin
6	Whey component
7	Whey component—also α-casein
8	Whey component
9	Immune globulins

the most heat-stable of the whey proteins. Their heat stability would identify them as belonging to the proteose-peptone fraction of milk (7). After the immune globulins, β-lactoglobulin is the next most heat-labile of the whey proteins. As indicated in Figure 1, β-lactoglobulin (Component 2) mostly disappears from the electrophoretic pattern of NSN of milk heated to 75 C. By 80 C it is all gone. α-Lactalbumin and serum albumin are still present at near their original concentration in the NSN fraction from milk heated to 85 C, and their boundaries are still perceptible in milk heated to 94 C. In the so-called pure preparations of these two components, the α-lactalbumin preparation contains some serum albumin as an impurity, whereas the pure serum albumin contains an unidentified second component. Component 1 is the tracking stain reagent used in the electrophoretic procedure; however, other material frequently appears to be associated with this boundary. As indicated by Table 1, concentration of NSN is lowest in the temperature range 90 to 103 C; at some temperature between 103 and 110 C, the concentration of NSN begins to increase again. At 117 C the electrophoretic patterns of Figure 1 indicate the NSN to consist principally of Components 4 and 7.

In Table 3 is presented the nitrogen distribution as determined by the Rowland procedure (12) of the NSN from commercial skimmilk and from two commercial evaporated milks. These data indicate that approximately 70% of the NSN from the evaporated milks is casein. The electrophoretic pattern of NSN from milk heated to 117 C, Figure 1, G, shows two components which migrate somewhat closer together than do α- and β-casein from unheated milk.

The effect of concentration of SNF on the amount of NSN produced by heating to 118 C for 10 min is shown in Figure 3. At concentrations of SNF higher than about 2:1 concentration ratio (above about 1.2% total nitro-

TABLE 3

Nitrogen distribution in two commercial evaporated skimmilks and one commercial skimmilk as determined by the method of Rowland (12) and by centrifugation[a]

	Commercial skimmilk	Evaporated skimmilk A	Evaporated skimmilk B
		(%)	
Total nitrogen	0.524	1.20	1.20
Nonsedimenting nitrogen	.138	.472	.486
Noncasein nitrogen	.110	.129	.157
Nonprotein nitrogen	.027	.086	.082
Per cent of total nitrogen as:			
Nonsedimenting nitrogen	26.3	39.3	40.5
Noncasein nitrogen	21.0	10.75	13.1
Nonprotein nitrogen	5.15	7.15	6.84
Per cent of nonsedimenting nitrogen as:			
Noncasein nitrogen	79.6	27.2	32.3
Nonprotein nitrogen	19.6	18.0	16.9

[a] The method of determining nonsedimenting nitrogen is described in the text.

gen) partial coagulation of the casein was brought about by the heat treatment. Rate of the coagulation interaction is more concentration-dependent than is the rate of the NSN-producing reaction. The increase in NSN formed with increasing concentration of the milk heated means that interaction between protein constituents occurs during formation of NSN. If there were no interaction, the per cent of total nitrogen as soluble nitrogen would be a constant value at all concentrations.

The effect of added phosphate on several properties of the caseinate system is shown in Figure 4. Added phosphate is seen to have, before heating, no effect on the amount of NSN; after heating, the amount of NSN increases in a regular manner with the concentration of added phosphate. The difference between heated and unheated milk with respect to added phosphate strongly implies that in unheated milk no reaction between the added phosphate and the soluble calcium has occurred. Calculations by Smeets (13) suggest that 90% of the soluble phosphate normally present in milk is unbound by calcium.

In Figure 5 is shown the effect of added citrate on the Ca : N ratio and the amount of NSN both before and after heating. In contrast to added phosphate, added citrate causes an increase in the amount of NSN both before and after heating. Before heating, part of the NSN is attributable to the whey proteins so that the values for NSN before and after heating, although similar in magnitude, do not represent similar nitrogenous components. The increase in NSN with increase in added citrate
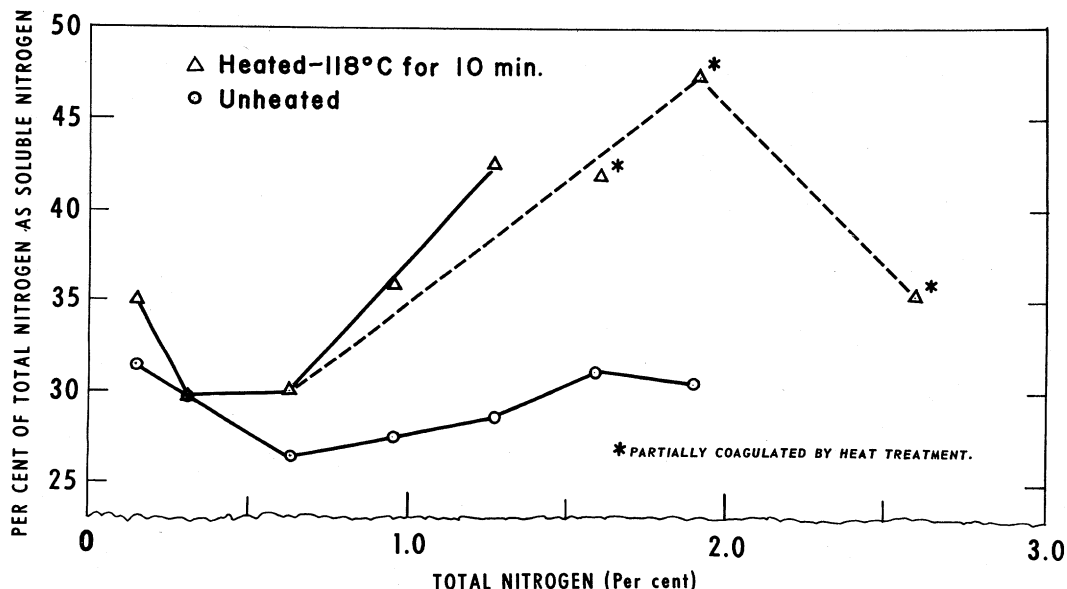

FIG. 3. Effect of concentration of solids-not-fat on amount of soluble nitrogen produced by heating to 118 C for 10 min.
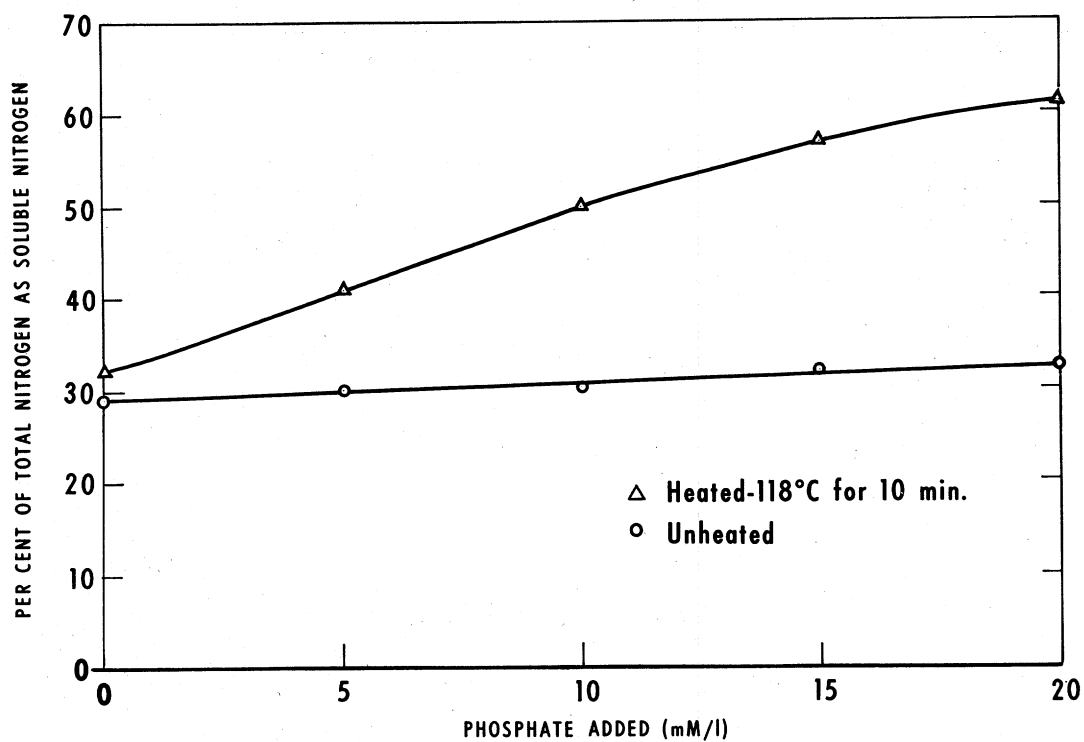
FIG. 4. Effect of added phosphate on production of nonsedimenting nitrogen from skimmilk before and after heating to 118 C for 10 min.
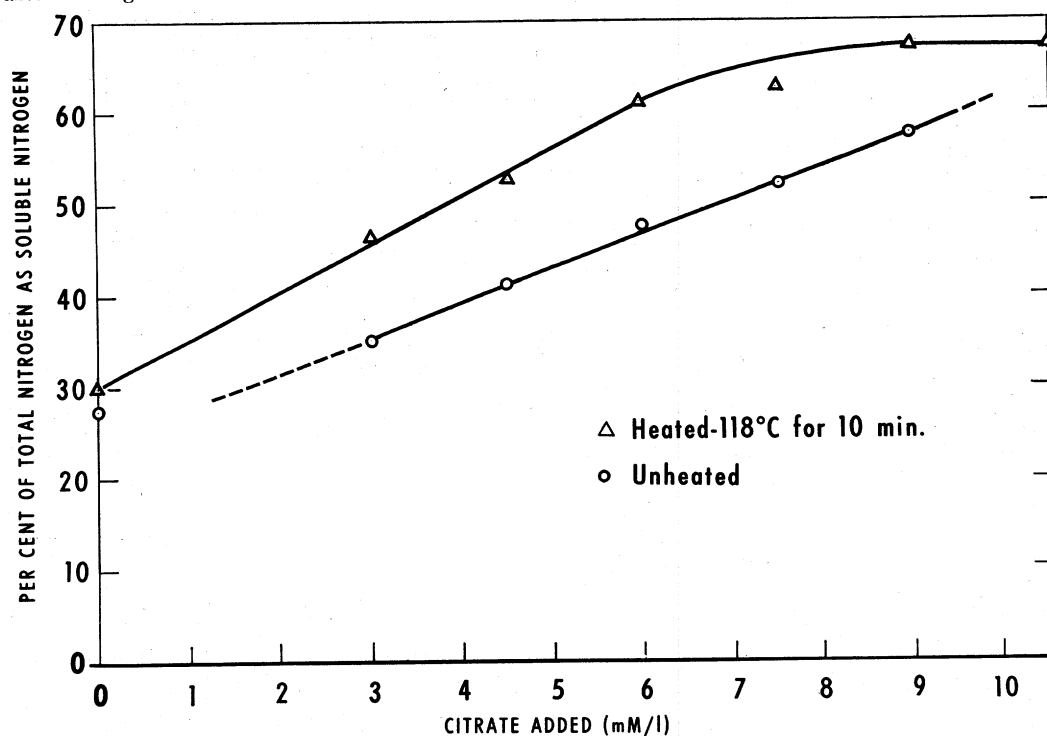

FIG. 5. Effect of added citrate on production of nonsedimenting nitrogen from skimmilk before and after heating to 118 C for 10 min.
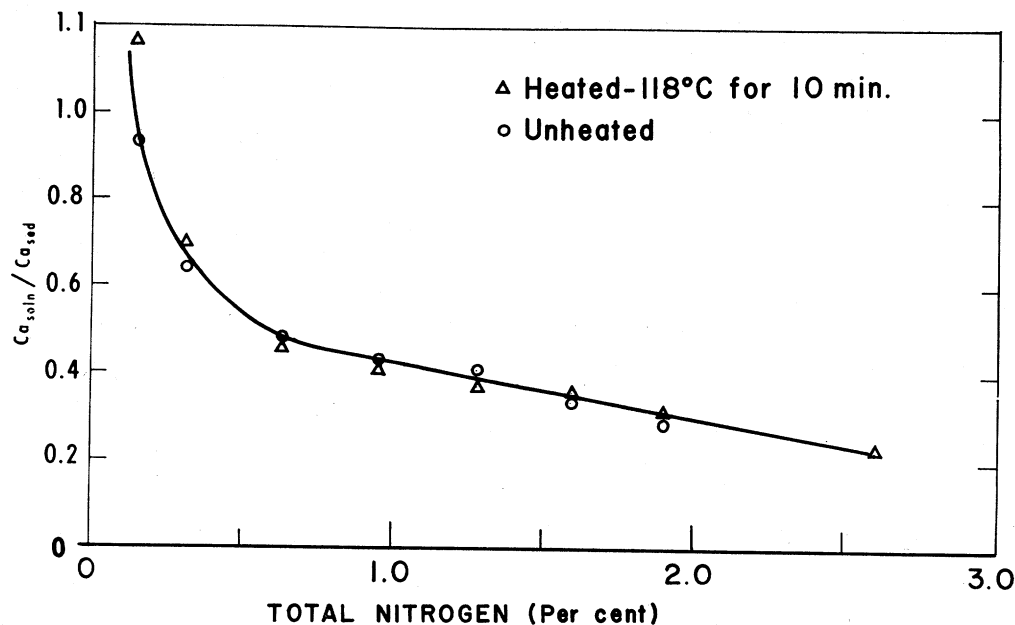
FIG. 6. Effect of concentration of solids-not-fat on distribution of calcium between the supernatant (Ca_{soln}) and sediment (Ca_{sed}) for both heated (after cooling) and unheated systems. The ratio is of the concentration of calcium in per cent.

in both heated and unheated milk is at the expense of the caseinate aggregates.

Distribution of calcium between the supernatant and sedimenting phases of both unheated and heated (after cooling) milk systems of different solids-not-fat concentrations is shown in Figure 6. The almost identical distribution of calcium in the heated (118 C for 10 min) and unheated systems is to be noted. The same shape of curve and the same coincidence of data for both heated and unheated milks exist whether there is plotted the ratio of concentrations, as is done in Figure 6, or the ratio of total calcium in supernatant to that in sediment.

When milk dialysate is heated, a considerable precipitate is formed. Analysis of the filtrate of these solutions (Table 4) shows that the citrate ion is not precipitated by the heat treatment. Almost identical results are obtained when a synthetic sera is heated under the same conditions. The only ions precipitated by the heat treatments are calcium and phosphate. Further, at room temperature, the calcium must exist as a soluble complex with citrate ($Ca\ Cit=$) because, in the absence of citrate, calcium and phosphate at their concentrations in milk dialysate immediately precipitate.

The occurrence of soluble caseinate in evaporated milk indicates that once the caseinate aggregates are dissociated, they do not readily reaggregate. Dialysis of decalcified milk against normal raw milk also did not result in any

TABLE 4

Effect of heating on solubility of calcium and phosphorus in milk dialysate and on a synthetic solution of similar salt content

| Heat treatment (C) | Milk dialysate | | |
| | Cal-cium | Ci-trate | Phos-phate |
	(mM/liter remaining in solution after heating)		
10 min at:			
None	9.6	8.5	12.4
48	8.6	8.5	12.4
55	7.4	8.5	10.8
65	6.9	8.5	10.3
75	4.9	8.5	9.2
90	3.7	7.9	8.1
15 min at:			
118	2.1	8.0	6.8
Synthetic sera[a]			
10 min at:			
None	10.3	10.2	14.7
55	6.4	10.2	11.6
65	5.4	10.2	11.6
75	5.3	10.2	10.5
85	5.2	10.2	10.3
94	4.5	10.2	9.7
15 min at:			
118	2.1	9.7	7.3

[a] Synthetic sera composition: 10 mM citrate, 10 mM calcium, 2 mM magnesium, 15 mM phosphate—1:1 ratio of KH_2PO_4 and K_2HPO_4.

reaggregation of the caseinate system. An average of three such experiments showed uptake of calcium by the dialyzing solution to be 58 mg

Ca/100 g solution. Distilled water dialyzed against milk took up 41 mg Ca/100 g water. The milk in which the samples were dialyzed contained about 121 mg Ca/100 g. The difference in calcium taken up by water and by the dialyzing decalcified milk represents calcium bound by the latter, but which did not cause reaggregation. The difference in calcium content between the dialyzing milk and the milk in which it is dialyzing is the fraction of total calcium, about one-half, responsible for the caseinate aggregates. These data indicate that the original caseinate aggregate or micelle is not a permanent entity, and when dissociated it is not restored to its original state by dialysis against normal milk.

Discussion
The identity of the nonsedimenting nitrogen in evaporated milks is established as being principally caseinate by its electrophoretic properties on polyacrilamide gel. Distribution of the NSN as measured by the Rowland procedure (12) is in agreement with the electrophoretic data.

Since dissociation of caseinate aggregates ordinarily occurs because calcium is removed from them, the effect of heating must likewise be to remove calcium from the aggregates. The experiments with added phosphate suggest that this ion under the influence of heat reacts with the calcium of the caseinate aggregates. That such reactions can occur in the salt system of normal milk is shown by the results of heating milk dialysate. Such heating precipitates calcium in the form of several calcium phosphate salts and leaves the citrate ion free in solution. Since, in the absence of the citrate ion, calcium and phosphate at the concentration they exist in the milk sera readily precipitate at room temperature, the calcium not in the caseinate aggregates must normally exist as a soluble calcium citrate complex. A change in the distribution of the calcium in milk as a consequence of heating has been noted by a number of investigators. Rose and Tessier (11), from the analysis of hot ultrafiltrates of milks heated to temperatures up to 110 C, found a 50% reduction in soluble (filterable) calcium. Evenhuis and DeVries (4) have suggested that on heat treatment, calcium and phosphate are transformed irreversibly into insoluble hydroxyapatite. Kannan and Jenness (6) state that when heated milk is aged some of the original colloidal calcium and phosphate dissolves to restore the content of dissolved calcium and phosphate to near their original levels in unheated milk. In Figure 6, the almost identical distribution of calcium in the heated and un-

heated systems points to the possibility of a complexing agent for calcium in the supernatant, which is not altered by heating.

Such a complexing agent is the citrate ion. According to Boulet and Marier (2) milk ultrafiltrate is normally saturated with tricalcium citrate. Removal of calcium from the citrate ion by formation of an insoluble calcium phosphate compound by heating, as happened when both milk dialysate and a synthetic sera were heated (Table 4), leaves the citrate ion free in solution. The pK value for either tricalcium citrate or $CaCit^-$ (2) is too great for citrate ion to remain free in solution. This is shown by the data of Figure 5 for the increase in amount of NSN as a function of citrate concentration in both heated and unheated milks.

Data of Table 4 show that heating milk dialysate or an aqueous solution of similar composition displaces calcium from a soluble citrate complex to form an insoluble precipitate with the phosphate present. Absence of such precipitates in heated milk indicates that heating causes the calcium and phosphate to be bound by the protein system so that a precipitate of the two ions is not formed. No NSN of predominantly caseinate composition is formed at pasteurizing temperatures, although the data of Table 4 show that both calcium and phosphorus (but not citrate) are precipitated from milk dialysate at these temperatures. Binding of calcium and phosphorus by the milk proteins must be a readily reversible and temperature dependent process until some critical temperature and time of heating is attained. Above this temperature, binding of calcium and phosphate may no longer be reversible upon cooling, and at this point formation of NSN from caseinate aggregates by removal of their calcium by citrate ion could commence.

References
(1) Association of Official Agricultural Chemists. 1960. Official Methods of Analysis. 9th ed. Washington, D. C.
(2) Boulet, M., and Marier, J. R. 1960. Solubility of Tricalcium Citrate in Solutions of Variable Ionic Strength and in Milk Ultrafiltrates. J. Dairy Sci., 43: 155.
(3) Edmondson, L. F., and Tarassuk, N. P. 1956. Studies on the Colloidal Proteins of Skimmilk. I. The Effect of Heat and Certain Salts on the Centrifugal Sedimentation of Milk Proteins. J. Dairy Sci., 39: 36.
(4) Evenhuis, N., and DeVries, Th. R. 1957. The Condition of Calcium Phosphate in Milk. III. Netherlands Milk Dairy J., 11: 111.
(5) Glick, David, ed. 1956. Methods of Biochemical Analysis, Vol. 3, p. 3. Interscience Publishers, New York.

(6) Kannan, A., and Jenness, R. 1961. Relation of Milk Serum Proteins and Milk Salts to the Effects of Heat Treatment on Rennet Clotting. J. Dairy Sci., 44: 808.

(7) Larson, B. L., and Rolleri, G. D. 1955. Heat Denaturation of the Specific Serum Proteins in Milk. J. Dairy Sci., 38: 351.

(8) Marier, J. R., and Boulet, M. 1958.. Direct Determination of Citric Acid in Milk with an Improved Pyridine–Acetic Anhydride Method. J. Dairy Sci., 41: 1683.

(9) Ramsdell, G. A., and Hufnagel, C. F. 1953. Effect of Heat on the Physical and Chemical Composition of Skimmilk. Proc. XIIIth Intern. Dairy Congr., The Hague, 3: 1025.

(10) Ramsdell, G. A., and Whittier, E. O. 1953. The Effect of Heat on the Albumin and Globulin in Milk. J. Dairy Sci., 36: 437.

(11) Rose, D., and Tessier, H. 1959. Composition of Ultrafiltrates from Milk Heated at 80 to 230 F in Relation to Heat Stability. J. Dairy Sci., 42: 969.

(12) Rowland, S. J. 1938. The Determination of the Nitrogen Distribution in Milk. J. Dairy Research, 9: 42.

(13) Smeets, W. Th. G. M. 1955. Determination of the Concentration of Calcium Ions in Milk Ultrafiltrate. Netherlands Milk Dairy J., 9: 249.

(14) Sullivan, R. A., Hollis, R. A., and Stanton, E. K. 1957. Sedimentation of Milk Proteins from Heated Milk. J. Dairy Sci., 40: 830.

(15) Yalman, R. G., Bruegemann, W., Baker, P. T., and Garn, S. M. 1959. Volumetric Determination of Calcium in Presence of Phosphate. Anal. Chem., 31: 1230.